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Purification of Influenza Virus Polypeptide Antigens and Studies of their Immunogenicity and Toxicity

ANNUAL PROGRESS REPORT

by

Edwin D. Kilbourne, M. D.
D. Bucher, Ph.D.

September, 1975

(For the period 31 July 1974 - 1 August 1975)

Supported by
U. S. Army Medical Research and Development Command
Office of the Surgeon General, Washington, D. C. 20314

Contract No. DADA17-69-C-9137

Mount Sinai School of Medicine
of the City University of New York 10029


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20. ABSTRACT - (Continued)

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A modification of the Weber and Kuter technique for the renaturation of enzymes has ~~enabled us~~ * to remove residual SDS from the hemagglutinin polypeptides with restoration of both biologic and immunogenic activity. ~~Most important,~~ removal of SDS has permitted the beginning of automated amino acid sequence analysis of the viral hemagglutinin. This is a major accomplishment and only one other group has thus far been successful in beginning the analysis of these sequences. Obviously this type of analysis will make it possible to identify precisely the nature of antigenic variation in relation to the amino acids involved and base coding.

Purification of specific antibodies to the hemagglutinin and its cleavage products has been achieved by immobilization of the antigens on agarose columns. Such specific antibody will facilitate our investigations of the biologic and cytotoxic activity of the various polypeptides.

Using another approach, the hemagglutinin and neuraminidase antigens have been isolated together by extraction following Triton X-100 disruption. This procedure allows hydrophobic reassociation of the two glycoproteins to enhance their antigenicity and again offers promise as a true subunit vaccine.

In an <u>in</u> <u>vitro</u> translation system, we have verified that influenza virion RNA is not the message and we have tentatively identified peptides produced <u>in</u> <u>vitro</u> by virus cellular RNA as NP, M and NS proteins.

In studies conducted by others of our neuraminidase specific antigenically hybrid vaccine, X-38, our investigation of the serologic response of human subjects has demonstrated the antigenicity of the preparation and the occurrence of "original antigenic sin" mediated through the neuraminidase.

* made it possible

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Science - National Research Council

SUMMARY

During the past year we have continued to improve our technique for the isolation and purification of the influenza viral polypeptides with special reference to the neuraminidase and hemagglutinin glycoprotein antigens. A very important finding has been the fact that biologically active and antigenically functional polypeptides can be isolated from influenza vaccine batches which provide us with a virtually unlimited source of material. Furthermore, using authenticated vaccine as a starting material prepared in accord with BOB specifications anticipates the use and acceptance of the purified antigens as vaccines.

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Purification of neuraminidase and hemagglutinin from vaccine virus preparations - potential use in subunit vaccines
(D. Bucher)

Applying the chromatographic techniques for separation of the polypeptides of influenza virus developed by Bucher (1975) and detailed in our progress report of last year, we have been able to purify both major surface antigens, the hemagglutinin polypeptides, (as HA_1 and HA_2) and the neuraminidase, as an enzymatically active component from a batch of influenza vaccine virus of the recombinant strain, X-38.

This finding is of considerable importance for two reasons: 1) the starting material has been prepared in accordance with BOB specifications for manufacture of vaccine, and 2) it introduces the prospect of using outdated or extra vaccine lots as valuable sources of purified antigens. Apparently recovery and isolation of the proteins in antigencially active form is not impeded by prior formalin inactivation.

Purification of the viral neuraminidase

Purification of influenza viral neuraminidase in mg quantitites provides both a useful biological reagent for investigators studying the effect of modification of cell surfaces by the removal of neuraminic acid and also results in the production of sufficient protein for use as an immunizing agent against influenza. The concept of a neuraminidase-specific infection permissive vaccine (E. D. Kilbourne, et al., 1972; Couch, et al., 1974) can be tested with the purified neuraminidase. Use of purified neuraminidase rather than recombinants bearing the strategic neuraminidase and "irrelevant" hemagglutinin should overcome any problems of cross-reactivity of the "irrelevant" hemagglutinin with the current hemagglutinin of the target virus. (see Neuraminidase-specific immunization of man, etc., below)

The neuraminidase component is purified as an enzymatically active component by affinity chromatography using columns to which the small organic compound, aminophenol

oxamic acid is bound. (Cuatrecasas and Illiano, 1971; Bucher, 1975; and earlier progress report) The aminophenoloxamic acid is an inhibitor for neuraminidase. Active neuraminidase molecules in a preparation of disrupted virus interact with the column while all other viral polypeptides pass through the column. The viral neuraminidase is eluted by elevation of pH. The remainder of the viral protein passes directly through the column. Neuraminidases have been purified from all influenza viral subtypes examined, including H0N1, H1N1, and H2N2 strains, recombinants of A strains, B strains and even from the paramyxoviruses, NDV and Sendai.

Starting with the X-38 (Heq1N2) vaccine and centrifuging the virus at 20,000 rpm for 90', approximately one half of the viral protein is recovered in the pellet. This viral protein was disrupted for application to the affinity column.

Taking the average of 10 preparations of enzyme from X-38 vaccine, 1.6 mg protein was recovered as viral enzyme when 150 mg of disrupted vaccine virus was passed through the column (2.5 X 12 cm). Approximately one-half of the enzyme activity was not adsorbed; generally the capacity of the column was exceeded with this quantity of influenza viral protein. A column of twice the bed volume would have permitted recovery of most of the enzyme activity in the vaccine preparation. Thus, approximately 1% of the viral protein applied was recovered as protein associated with the enzyme. The eluted enzyme was recovered in a 20-30 ml volume with protein concentrations averaging 0.06 mg/ml. The specific enzyme activity of the 10 preparations averaged 25.4 μM/min/mg (range of 8.0 to 49.5 μM/min/mg.) The average increase in specific activity or fold purification was 10.6 (range of 3.5 to 21.4).

The enzyme purified from the vaccine was quite stable. Only slight inactivation could be detected when the enzyme

was incubated overnight at 37° in a pH 5.9, 0.1M phosphate buffer as compared with a 15 min. incubation at 37°.

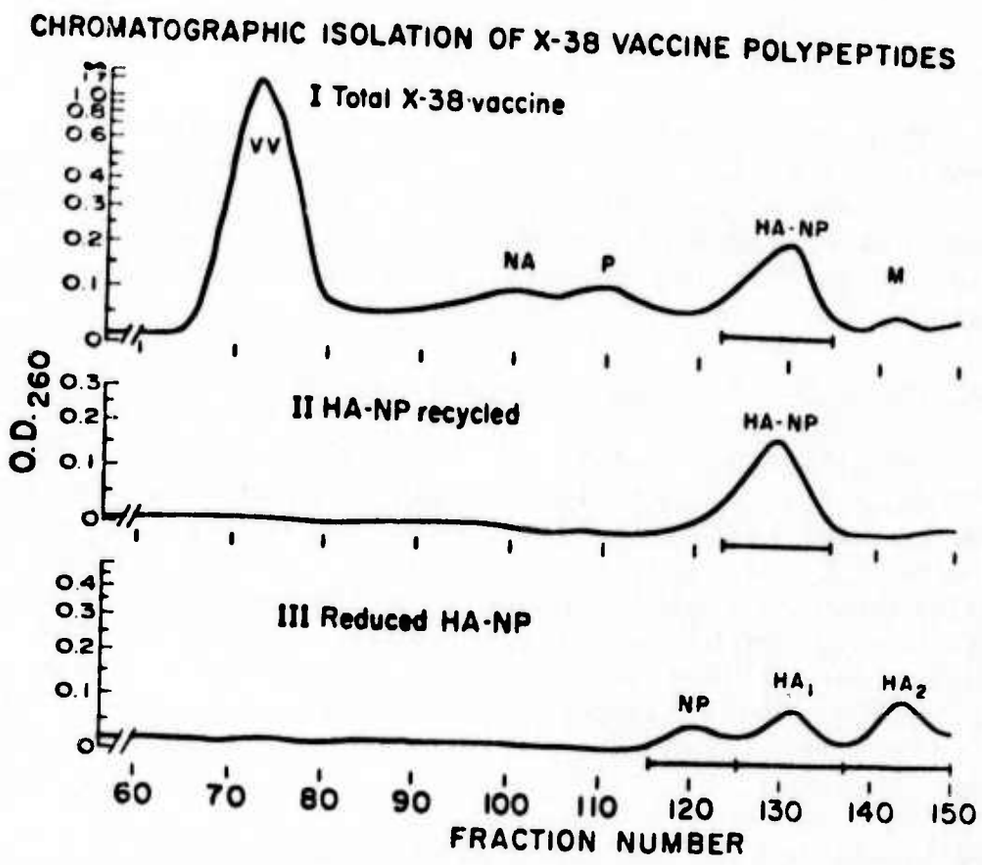
As prepared, the enzyme contains 0.1% Triton X-100. Evidently the Triton X-100 is not essential for stabilization of activity once the enzyme has been purified. Dilutions of the enzyme by a factor of 1:1000 in buffer not containing Triton X-100 did not result in any detectable inactivation. If necessary for vaccine purposes, the Triton X-100 could probably be removed by a single chromatographic technique using styrene-divinyl benzene copolymer beads such as Bio-Beads SM2 (Holloway, 1973).

Purification of the hemagglutinin polypeptides

Viral hemagglutinin as HA_1 and HA_2 was purified either from the non-adsorbed viral proteins from affinity chromatography or directly from the vaccine without prior removal of the neuraminidase. The hemagglutinin is synthesized as a glycoprotein of approximately 75,000 daltons. Proteolytic cleavage of the hemagglutinin by host proteases *in ovo* is asymmetric and results in the production of two subunits which are held together by disulfide linkage. These subunits are HA_1 which is 50,000 and HA_2 which is about 25,000. This characteristic of the structure of the hemagglutinin of virus grown *in ovo* permits separation by gel filtration under reducing and non-reducing conditions. Chromatography without reduction of the viral proteins separates the hemagglutinin (HA) from all viral proteins with the exception of the nucleoprotein (NP)(Bucher, 1975). Reduction of the HA-NP fraction and chromatography results in the purification of NP, HA_1 and HA_2. When 300 mg of total viral vaccine protein was applied to the column at the first stage (see Figure 1), 20-22 mg. (7%) was recovered as NP, 32-38 mg (11-13%) as HA_1 and 12-20 mg (4-7%) as HA_2 at the third chromatographic cycle. From the compilation on percentage composition of the polypeptides of the influenza virus in the review by Schulze (1973) for those viruses with the total hemagglutinin

Figure 1.

Chromatography of X-38 vaccine viral polypeptides



present as HA_1 and HA_2, HA_1 should represent 19-21% and HA_2 should be 12-15% of the total protein. NP should represent 26-31% of total viral protein. Thus we have recovered approximately one-half of the available hemagglutinin polypeptides and approximately one-fourth of the NP present in the vaccine as the purified products. These recovery estimates are certainly low because they assume a purity of 100% virus in the vaccine preparation. Cellular protein brought along through the purification of the virus probably dimishes this value considerably.

This chromatographic technique also purifies mg quantitites of NA, P and M (in addition to resulting in purification of HA_1, HA_2, and NP). All of the influenza viral polypeptides can be purified from the same viral batch in quantitites permitting sophisticated structural analysis.

Removal of SDS from the hemagglutinin polypeptides

By slight modification of the Weber and Kuter (1971) technique for renaturation of enzymes once denatured by SDS, we have been able to successfully remove this anionic detergent by means of a Dowex-1 anion exchange resin. The application of this technique to influenza viral hemagglutinins has two highly significant implications: 1) the restoration of both biologic and immunogenic activity to the hemagglutinin polypeptides and 2) the removal of SDS permits automatated amino acid sequence analysis of the viral hemagglutinins.

Immunogenicity of "renaturated" HA_1 and HA_2:

Immunogenicity of SDS denatured hemagglutinin polypeptides as HA_1 and HA_2 has been shown to be very low (Erickson and Kilbourne unpublished results). The purified, "renatured" HA_1 and HA_2 polypeptides are now being used as immunogens in rabbits and we should soon see how effective these "renatured" polypeptides are in raising an antibody

response against the hemagglutinin of intact virus. If the antigenicity to hemagglutinin resides in HA_1 (and HA_2 serves merely to anchor the hemagglutinin into the envelope) as proposed by Brand and Skehel (1972) and also Ecker (1973), then one could envision subunit vaccine preparations which would consist only of HA_1 protein. Our own observations with studies of immobilized HA_1 and HA_2 would suggest that HA_2 also plays a role in determining the antigenicity of the hemagglutinin, see below (Bucher and Kilbourne, unpublished results).

Amino acid sequence analysis of influenza viral hemagglutinin

A recent, exciting development in our study of the viral hemagglutinin has been the successful sequence analysis by Dr. Steven Li of the N-terminal residues of HA_1 and HA_2 purified from a batch of vaccine which is virus which has been formalin inactivated. It has been reported that formaldehyde is capable of modifying several different amino acids. Evidently the conditions of formalin inactivation of the virus for vaccine production did not introduce modifications of amino acids of sufficient significance to interfere either with the purification or automatic sequencing of these polypeptides.

Dr. Li has shown that the amino-terminal sequences of hemagglutinin HA_1 and HA_2 subunits of equine type 1 (X-38) are clearly homologous to the partial sequences of type A subgroups (H0, H1, H2 and H3) and type B (B/Lee) of human influenza virus in the recent earlier work of Skehel and Waterfield (Figure 2). The amino-acid sequence of the first 10 residues of HA_1 of equine type 1 has 3 to 4 differences from those of human type A subgroups (H0, H1 and H2), and these differences can be explained by single nucleotide substitutions. The amino-acid sequence of the first 10 residues of equine 1 HA2 is identical to those of human Type A subgroups (H0, H1 and H2 and H3) of influenza virus.

HA1

		1				5				10	
X-38	(Heq1)	Asp	Lys	Ile	Ser	Leu	Gly	Tyr	His	Ala	Val-
Bel	(HO)	-	Thr	-	-	Ile	-	-	-	-	Asx
Weiss	(H1)	Asn	Thr	-	-	Ile	-	-	-	-	Asx
Singapore	(H2)	Asn	Glu	-	-	Ile	-	-	-	-	Asx

Skehel and Waterfield (1975)

HA2

		1				5				10			15				20			24
X-38	(Heq1)	Gly	Leu	Phe	Gly	Ala	Ile	Ala	Gly	Phe	Ile	Glu	Asn	Gly	Trp	Glu	Gly	Leu	Ile	Asp	Gly-	?	-Tyr	Gly	Tyr-
Bel	(HO)	-	-	-	-	-	-	-	-	-	-	Glx	Gly-												
Weiss	(H1)	-	-	-	-	-	-	-	-	-	-	Glx	Gly-												
Singapore	(H2)	-	-	-	-	-	-	-	-	-	-	Glx	Gly												
X-31	(H3)	-	-	-	-	-	-	-	-	-	-	Glx	Asx												
MRC-11	(H3)	-	-	-	-	-	-	-	-	-	-	Glx	Asx-												
B/Lee		-	Phe	-	-	-	-	-	-	-	Leu	Glx	Gly-												

Skehel and Waterfield (1975)

Fig. 2 Amino-terminal sequences of influenza virus hemagglutinin subunits

It appears that the amino-acid sequences of HA_2 subunits are more conserved than those of HA_1 subunits. This is consistent with the antigenic variation of hemagglutinin subunits. The partial sequence of HA_2 of equine type 1 (X-38) seems to be more similar to those of human type A subgroup H3 (X-31 and MRC-11) than other subgroups (H0, H1 and H2). The asparagine-glycine difference at residue no. 12 can only be explained by two nucleotide replacements. It may also be noted that the HA_2 of human type B influenza virus has two amino acid differences at residue no. 2 and 10 out of the first 10 residues compared with those of type A influenza viruses, either human subtypes H0, H1, H2 or H3 human type A subgroups and/or Heq.1.

The amino-acid sequence of the first 23 residues of HA_2 hemagglutinin shows several features of interest: 1) a pseudo-palindrome of 7 amino acids centered on the isoleucine at position 6, first observed by Skehel, is confirmed; 2) almost one-third of 23 residues identified are glycine, although only about 10% of entire HA_2 are glycine, based on the data of Laver and Baker (1972), and Skehel and Waterfield (1975); 3) these glycine residues are regularly distributed, that is, every 3 or 4 residues are glycine residues. If this portion of HA_2 is an α-helix, this finding suggests a spatial constraint for one side of the helix, as the glycyl residues would have no side chain.

Although this limited information points to greater changes in the HA_1 than in the HA_2 from the other hemagglutinin polypeptides sequenced from the amino acid terminal end, sequence of the entire HA_1 and HA_2 polypeptides must be performed for any comparisons to be made. With sequence analysis we will be able to precisely relate changes in the amino acid sequence. Given a technique for purification of the polypeptides, which provides large quantities of viral polypeptides it should be possible to accomplish full sequence analysis of the polypeptides of influenza viral hemagglutinin.

Purification of specific antibodies to HA, HA_1 and HA_2

Hemagglutinin polypeptides from X-38 which had been purified by SDS gel filtration and renatured via the Weber and Kuter technique were immobilized on agarose columns following CNBr activation of the agarose. Either HA_1, HA_2 (or mixture of HA-NP) were immobilized on the column. Antisera to the whole virus (X-38) was passed directly through the column and the column containing the bound antibodies was washed thoroughly with phosphate buffered saline. Elution occurred with elevation in the salt concentration.

Assay of the eluted antibodies showed that plaque inhibiting and hemagglutination inhibiting antibodies could be adsorbed and eluted from all three columns. However, when the eluted antibody solutions were tested for the presence of anti-neuraminidase antibodies, very little or no activity was found. Anti-neuraminidase antibodies had passed directly through the column. This indicates the specificity of the interaction between the antibodies and the polypeptides on the immobilized column.

It remains to be shown whether or not the antibodies which adsorb to the HA_1 column can or cannot be adsorbed to the HA_2 columns and conversely. Presumably, antibodies adsorbing to HA_1 columns should not adsorb to the HA_2 columns although both types of antibodies could cause hemagglutination inhibition and plaque inhibition.

Although these experiments are still quite preliminary, the results are significant in that we would now have a technique for pulling out specific antibodies to the hemagglutinin polypeptides (either HA_1 or HA_2) for study of antibody-virus interactions in tissue culture systems without the interference of other components of the antisera. In addition, it may be valuable to study the antigenicity of the hemagglutinin polypeptides in rabbits,

comparing 1) soluable HA_1 and HA_2 and 2) immobilized HA_1 and HA_2. It is conceivable that immobilization of the polypeptides may greatly enhance their antigenicity.

Another aspect of the immobilization of these polypeptides is the potential use of these columns, if the active site is retained, as lectin columns in the same manner as Concanavalin A immobilized on agarose. Conceivably, immobilized hemagglutinin, by virtue of its binding to neuraminic acid of glycoproteins, could be used for the purification of such glycoproteins.

Finally, the adsorption of plaque inhibiting and hemagglutination inhibiting antibodies to both immobilized HA_1 and HA_2 columns would dispute the present assumption among influenza virus investigators that all of the antigenicity to the hemagglutinin resides with HA_1 and that HA_2 exists as a kind of "anchor" for HA_1 (Brand and Skehel, 1972; Eckert, 1973). Thus, it is important to pursue these studies of the immobilized polypeptides to increase our understanding of the nature of the antigenicity of the hemagglutinin and to provide purified antibodies as a tool for studying virus-cell interaction.

Triton extracted influenza viral glycoproteins as antigens (Schulman and Palese)

Following techniques described by Scheid et al. we have tested the immunogenic potency of glycoproteins (hemagglutinin and neuraminidase) extracted from commercial vaccine (X-37) by triton. Briefly, the extraction procedure involves:

1) addition of triton X-100, 2% + 0.5 M KCl
2) stirring - 20 minutes at room temperature
3) sonication - 5 minutes
4) centrifugation, 40,000 RPM in 50 Ti rotor
5) removal of supernatant and repetition of steps 1-4
6) dialysis of supernatant vs. PBS
7) slow speed centrifugation
8) addition of 10 volumes of butanol
9) slow speed centrifugation
10) treatment of pellet with ether and then removal of ether by evaporation

Following these procedures, we were able to recover 38% of the protein in the starting material (HA + NA are estimated to comprise 30-40% of the viral protein), and 100% of the neuraminidase activity of the starting material. Hemagglutinating activity was also recovered in titers which exceeded those of the starting material, but since the HA activity depends on the state of aggregation of the proteins, the latter value is of no significance. The triton extracted pellet was resuspended in a volume equal to that of the starting material (20 ml) to compare its immunogenic activity with that of unextracted whole virus. Graded dilutions of each were inoculated into 5 rabbits/group and serum was obtained 10, 21 and 42 days after immunization for measurements of HI and NI activity. The results summarized in Tables 1 and 2 demonstrate that the antigenic dose/50 (the minimum dose required to elicit an HI antibody response) was identical for the 2 vaccines. Moreover, the mean HI antibody responses 42 days after immunization in recipients of the triton extracted glyco-

Table 1

Hemagglutinating Inhibiting Antibody Response in Rabbits Immunized with Intact X-37 Vaccines, or Triton Extracted Glycoprotein

Vaccine-Dose	10	21	42
Whole Virus undiluted	5.4*	5.8	3.4
1:5	3.4	3.8	2.4
1:25	3.0	2.6	1.0
1:125	<1.0	<1.0	<1.0
1:625	<1.0	<1.0	<1.0
Triton Extract undiluted	4.0	4.4	3.0
1:5	2.8	3.4	2.2
1:25	2.4	2.8	1.2
1:125	<1.0	<1.0	1.0
1:625	<1.0	<1.0	<1.0

*Serum HI antibody titer, geometric mean $\log_2$

Table 2

Neuraminidase Inhibiting Antibody Response in Rabbits Immunized with Intact X-37 Vaccine, or Triton Extracted Glycoproteins

Vaccine-Dose	Days After Immunization 10	21	42	
Whole Virus undiluted	5/5*	5/5	5/5	(40)**
1:5	2/5	2/5	2/5	(12)
1:25	0/5	0/5	0/5	
1:125	0/5	0/5	0/5	
1:625	0/5	0/5	0/5	
Triton Extract undiluted	1/5	5/5	5/5	(22.4)
1:5	1/5	2/5	5/5	(18.8)
1:25	0/5	1/5	1/5	
1:125	0/5	0/5	1/5	
1:625	0/5	0/5	1/5	

* Proportion of animals with NI antibody titers $\geq$ 1.4
**Arithmetic mean of NI antibody titer

proteins were equivalent to those observed in animals immunized with equivalent dilutions of whole virus. Similarly, although the NI response to the triton extracted material occurred later than in recipients of whole virus, the proportion of animals responding and the mean NI titers (shown by the figures in parenthesis in table 2) were equivalent 42 days after immunization.

These data provide evidence that influenza virus glycoproteins can be isolated from the virus by relatively easy procedures that might readily be adapted to commercial vaccine production, and that the proteins recovered are as antigenic (at least in rabbits) as the envelope antigens on undisrupted virus. One of the attributes of this extraction procedure is that the glycoproteins are removed with their hydrophobic ends intact. Following butanol extraction, aggregates of HA and NA are formed, a fact which may contribute to their retention of antigenicity.

Analysis of influenza viral protein made in vitro and in vivo (Ritchey, Palese)

In order to further characterize the replication process of influenza viruses, the question of whether or not influenza viral RNA was negative or positive for message was investigated. An in vitro translation system using wheat embryos was developed for this purpose. In the course of these studies it was necessary to analyze the polypeptide products of the in vitro reaction and in so doing to re-examine viral polypeptides made in vivo. This analysis was carried out by SDS gel electrophoresis using both 13% and 7-20% polyacrylamide gradient gels, and peptide mapping of tryptic digests. The following results were obtained.

RNA isolated from purified virions using strain PR8 grown in the allantoic cavity of embryonated eggs was found to give slight stimulation in the in vitro translation system, but the products were unlike any viral proteins when analyzed on gels. In contrast, RNA isolated from WSN virus-infected cells using MDCK, MDBK, and CEF monolayers gave greater stimulation and products that were similar to viral proteins on gels. The products were identified as NP, M, NS, P_1, and P_2. These results add further confirmation that influenza A is a negative strand virus in which virion RNA is not message.

A further comparison of the proteins made in vivo and in vitro was made by column chromatography of methionine labeled tryptic digests of NP, M, and NS proteins. It was found that all of the peptides of these proteins made in vivo were also made in vitro. In addition, it was noted that the NS protein had

more and different methionine-labeled tryptic peptides than the M protein. This latter finding, that M and NS are different, agrees with the results of Lazarowitz et al. (1971).

In the process of comparing the products made in vitro and in vivo on 7-20% gradient gels, it was noticed that the NP and NS usually appeared as doublets, and the M protein also became a doublet if the electrophoresis was carried out for a longer time. This may suggest that there are really two species of these proteins or that a post translational modification occurs. Analysis of tryptic digests of these doublets will be used to determine if any difference can be demonstrated between the members of a pair.

An additional finding was the fact that the M and NP proteins of PR8 and WSN (both HON1 strains) have slightly different migration rates in these gel systems. This was surprising in view of the fact that all A strains are generally assumed to have similar NP and M proteins; indeed, they are classified as type A strains on the basis of antigenic relatedness of their NP & M proteins. The differences in gel patterns noted here may reflect a real difference in these proteins of the two influenza A strain viruses. Further studies are planned to investigate this finding.

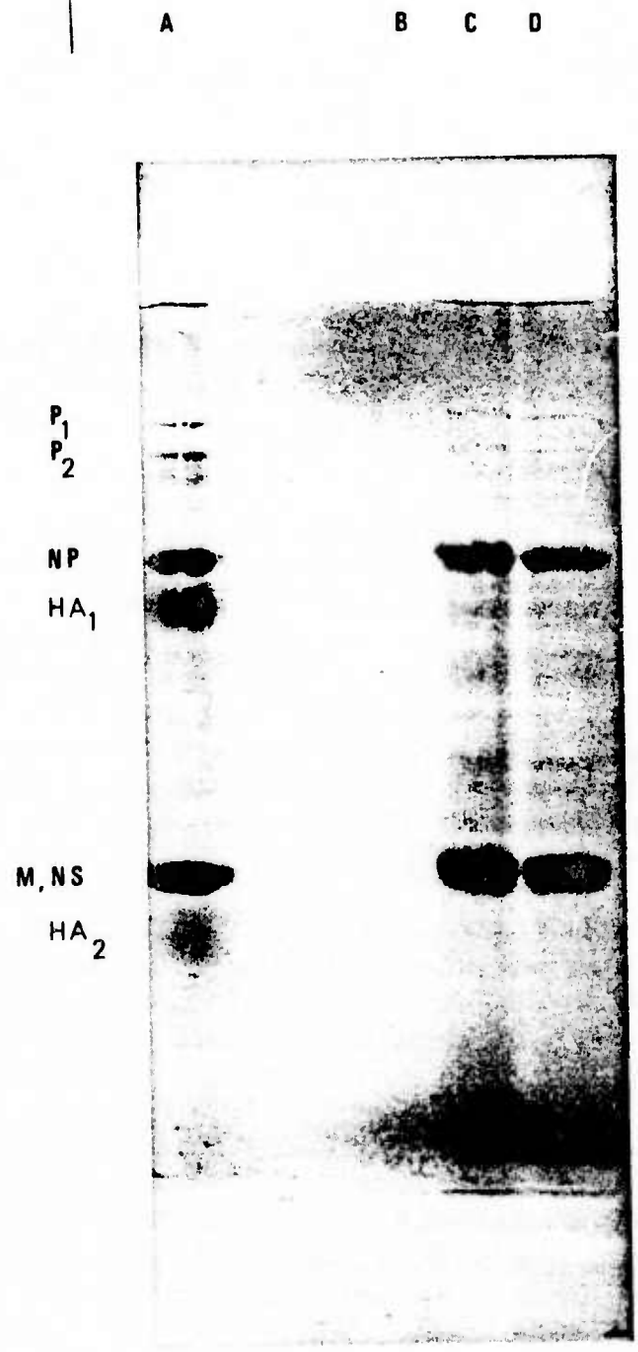


Figure 3. Influenza virus proteins translated in vitro. Proteins made in vivo and in vitro were electrophoresed on a 13% polyacrylamide SDS gel. A) purified WSN virus grown in MDBK cells (C^{14} amino acid label) B) in vitro assay minus RNA. C and D) in vitro assay plus RNA isolated from WSN virus-infected CEF monolayer. B, C and D are labeled with ^{35}S methionine.

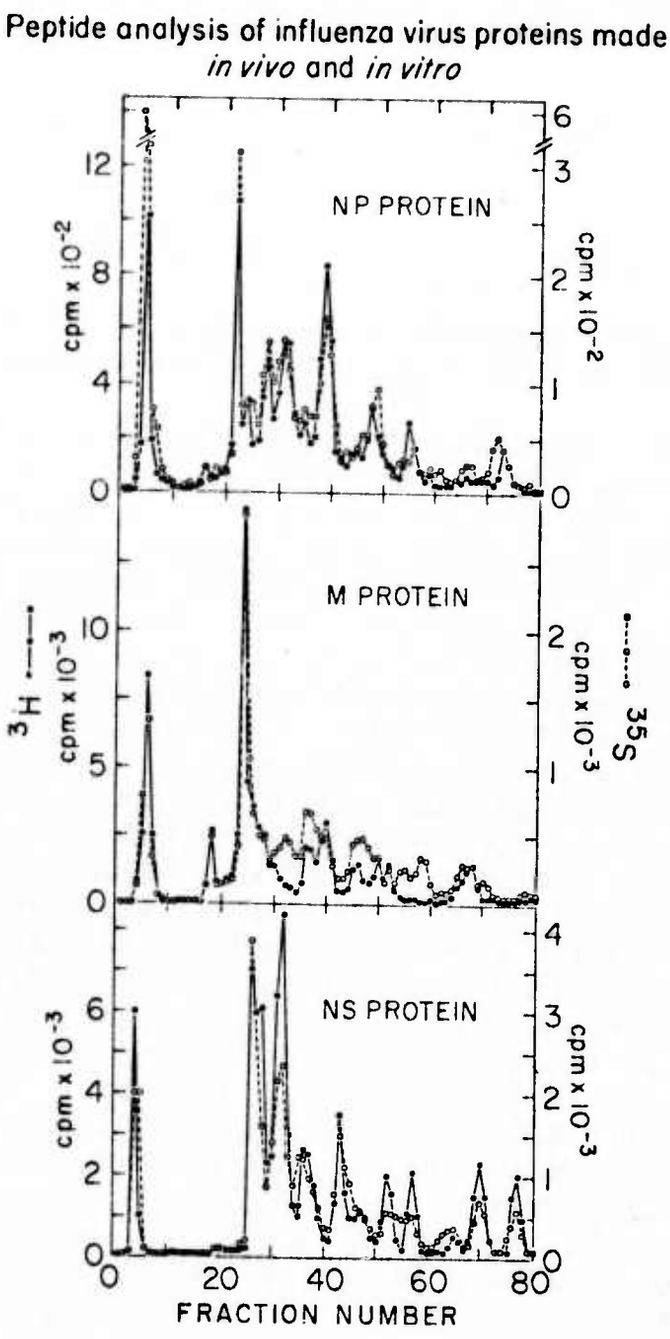


Figure 4. Peptide analysis of influenza viral proteins made in vivo and in vitro. WSN virus was grown in CEF monolayers and labeled with ^{3}H-methionine. RNA was isolated from WSN virus infected CEF monolayers and used in a cell-free wheat germ translation system to make S^{35} methionine labeled protein. Proteins made in the cell free system (in vitro) and in infected cells (in vivo) were separated on polyacrylamide SDS gels, electrophoresed from the gel fragments, digested with TPCK-trypsin, and applied to an ion exchange resin (Chrome Beads, Type P). The peptides were eluted using a gradient of pyridine-acetate from 0.2N (pH3.1) to 2.0N (pH5.0). Fractions containing the peptides were analyzed for ^{35}S content (in vitro) and ^{3}H content (in vivo).

Neuraminidase-specific immunization of man with an antigenically hybrid influenza virus vaccine (X-38)

In a study conducted by others (with serology done by us), groups of college students (30-39 per group) received either conventional A/England/42/72 (H3N2) vaccine (X-37), or an antigenically hybrid (HeqlN2) vaccine (X-38) containing the same neuraminidase or a placebo injection. The vaccines contained 798 and 643 CCA units per dose respectively, and equivalent immunogenic units of N2 as defined in antigenic extinction tests in rabbits. All subjects had preimmunization anti-N2 antibody and mean initial titers were comparable in both vaccine groups (1:56). Homotypic H1 responses to vaccine hemagglutinin occurred in 72% of X-37 and 77% of X-38 vaccinees. Initial H1 titers versus A/England/42/72 were low, (1:4 or less). Significant anti-N2 (N1) response was observed in 25% of X-37 and 69% of X-38 vaccinees - an unexpected finding. Mean N1 response was 2 fold greater in the X-38 group. Another unanticipated finding was the occurrence of heterotypic H1 response (i.e. versus H3) in 56% of those receiving X-38 vaccine. Homotypic H1 response was less in the X-38 than in the X-37 group. Testing of N1 response with earlier neuraminidase antigens demonstrated "original antigenic sin" from earlier priming involving also the N1 neuraminidase.

Enhanced cytopathic effect of influenza virus in interferon-treated cells (S. Mowshowitz)

Viruses inactivated with respect to infectivity may nevertheless retain certain of their biological properties. In the case of influenza virus, RNA transcriptase, neuraminidase and hemagglutinin activities may be minimally affected by treatments which destroy infectivity. Certain host responses may also be elicited by formalin or U.V.-inactivated influenza virus such as specific antibody formation. Interferon-inducing capacity is actually enhanced by U.V.-inactivation.

In addition to its well-known role in the establishment in cells of a state refractory to virus replication, interferon has a variety of other effects including inhibition of DNA synthesis in certain cells, suppression or enhancement of various limbs of both the humoral and cellular immune response, and antitumor activity.

An additional effect of interferon-treatment on cells is described here: a specific enhancement of the cytopathic effects of influenza virus. In its dual capacity as a potent cytopathic agent in interferon-primed cells and inducer of interferon synthesis, influenza virus may enhance its own cytopathogenicity.

Materials & Methods

Clone 1 5C-4 cells were cultivated in medium 199 supplemented with 10% fetal calf serum. After establishment of confluence, 0.2% bovine serum albumin was substituted for fetal calf serum.

X-31, Aichi and PR8 strains of influenza were grown in the allantoic cavity of chick embryo with an inoculum of approximately 10^3 EID_{50} per egg. VSV was grown in CEF cells. An initial m.o.i. of 0.01 was used.

NDV-induced human embryo kidney interferon was a product of Abbot Laboratories, N. Chicago, Ill. (Lot #3761-72-I CCS) and contained 16,000 PDD_{50} mZ^{-1} for VSV in the human amnion cells. Prior to use, the preparation was dialyzed first against 50mMolar KCl-HCl buffer pH 2.0 for 48 hours, and then against PBS overnight.

Cell viability was determined by neutral red uptake. Cell sheets were exposed to 1:30,000 neutral red solution in medium 199 for 2 hours at 37C. After two washes with PBS, the dye taken up was extracted with 100mMolar citric acid in 20% ethanol. Optical density of the extract was measured at 520nm.

Yields of viral neuraminidase were determined according to the method of Warrenhoff.

Results

Sensitivity of X-31 and Aichi virus replication in clone 1 5C-4 cells to interferon pre-treatment

Confluent sheets of human conjunctival cells were pretreated for 16 hours with various doses of interferon prior to challenge with either Aichi or X-31 virus at an m.o.i. of 0.1.

After 24 hours at 37C the viral yields were harvested and determined. The results appear in Table III.

TABLE III

SENSITIVITY OF AICHI AND X-31 VIRUS YIELDS TO INTERFERON

	X-31		AICHI	
Dose of Interferon[1]	Virus Yield[2]	% of Control Yield	Virus Yield	% of Control Yield
0	9114	100	3228	100
5	7672	84	2610	81
10	6355	70	1644	51
20	4792	53	860	27
40	3536	39	494	15

[1] PDD_{50} for VSV in human amnion cells
[2] Measured as viral neuraminidase, arbitrary units

Both Aichi and X-31 viruses are sensitive to interferon. Aichi appears to be somewhat more sensitive as can be seen from a graphical analysis of the data from Table III. (Figure 5).

Cytopathic effect of influenza virus in cells pre-treated with interferon

After pre-treatment of confluent cell sheets for 16 hours with various doses of interferon, a challenge m.o.i. of 3 was applied to the cells. After 22 hours of incubation at 37C the medium was removed and replaced with medium 199 containing 1:30,000 neutral red. Cell viability was determined as described. The results appear in Table IV.

A dose-dependent enhancement of the cytopathic effects of influenza virus by interferon is observed. Interferon alone, even at higher doses, manifest no cytopathic effect in these cells (Table V).

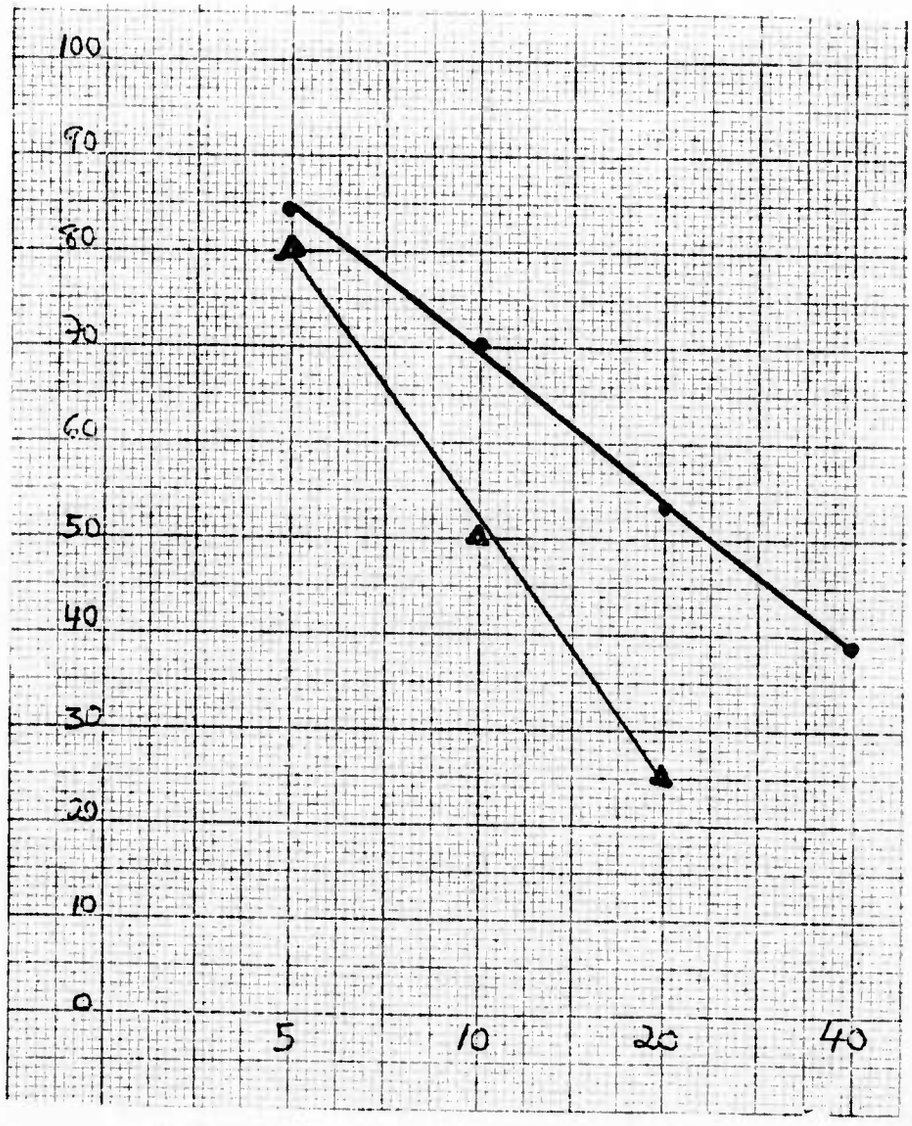
Effects of U.V.-irradiation on the cytopathic effects of influenza virus in interferon-treated cells

Clone 1 5C-4 cells were pretreated with 0 or with 20 units of interferon for 16 hrs. at 37C. They were then challenged at an m.o.i. of 3 with fully infectious Aichi virus, or an equivalent amount of U.V.-irradiated Aichi virus (5×10^{-3} Wcm^{-2} for 30"). After incubation for 22 hours at 37C, cell viability was determined as described. The results are presented in Table VI.

The U.V.-irradiated virus appears to exert no cytopathic effect in either interferon-treated or untreated cells.

FIGURE 5

Sensitivity of AICHI and X-31 Virus Yields to Interferon[1]



[1]Data derived for Table III

TABLE IV

Effect of Interferon Pre-Treatment on the CPE of X-31 and AICHI Viruses

Dose of Interferon[1]	X-31 % Surviving Cells[2]	AICHI % Surviving Cells
0	83	20
5	74	9
10	53	8
20	38	5
30	35	-

[1] PDD_{50} for VSV in human amnion cells

[2] Measured as neutral-red uptake as described

TABLE V

Lack of Cytotoxicity of Interferon for Human Conjunctival Cells

Dose of Interferon[1]	% Surviving Cells[2]
0	100
10	98.2
20	109
40	107
80	106
160	113

[1] PDD_{50} for VSV in human amnion cells
[2] Measured as amount of 2 hr neutral red uptake after 24 hr exposure to interferon.

TABLE VI

Effect of U.V.-Inactivation on CPE
of AICHI Virus in Interferon-Treated Cells

Treatment	% Surviving Cells
None	100
Interferon Pre-Treatment	100
AICHI Virus	17
U.V.-Irradiated AICHI Virus	88
Interferon-Pre-Treatment, U.V. Irradiated Virus	95

Comparison of NDV, X-31 and VSV in cytotoxicity for interferon-treated cells

After a 16 hour pre-treatment with 0 or 50 units of interferon, clone 1 5C-4 cells were challenged with an m.o.i. of 3 of either X-31, NDV or VSV virus. After 22 hours at 37C cell viability was again measured. The results constitute Table VII.

The enhancement of cytopathogenicity can be seen to be specific for influenza virus. Neither NDV nor VSV exhibit this effect. In fact, interferon pre-treatment appears to protect cells against the cytopathic effects of the latter.

Comparison of the effects of interferon pre-treatment on CPE in cells infected with PR8 or X-31 virus

After a 16 hour pretreatment with various doses of interferon, cells were challenged with an m.o.i. of 3 of either X-31 virus or an equivalent amount (normalized for H.A. units) of PR8 virus. (The latter undergoes an abortive replication cycle in clone-1-5C-4 cells.) After 22 hours of incubation at 37C, cell survival was determined (Table VIII).

Interferon appears to enhance the CPE of X-31 as is expected, but protects cells against the CPE of PR8. Figure 6 is a graphical representation of the data from Table VIII.

Discussion

Interferon pre-treatment appears to enhance the cytopathogenicity of influenza virus in clone 1-5C-4 cells. Interferon itself has no cytopathic effect in

TABLE VII

Effect of Interferon Pre-Treatment on CPE
of X-31, NDV and VSV

Virus	% Surviving Cells[1]-Interferon[2]	% Surviving Cells + Interferon
NDV	53	72
VSV	55	100
X-31	83	48
NONE	100	100

[1]Measured as neutral red uptake as described

[2]50 PDD_{50} for VSV in human amnion cells

TABLE VIII

Comparison of X-31 and PR8 Virus CPE in Interferon-Treated Cells

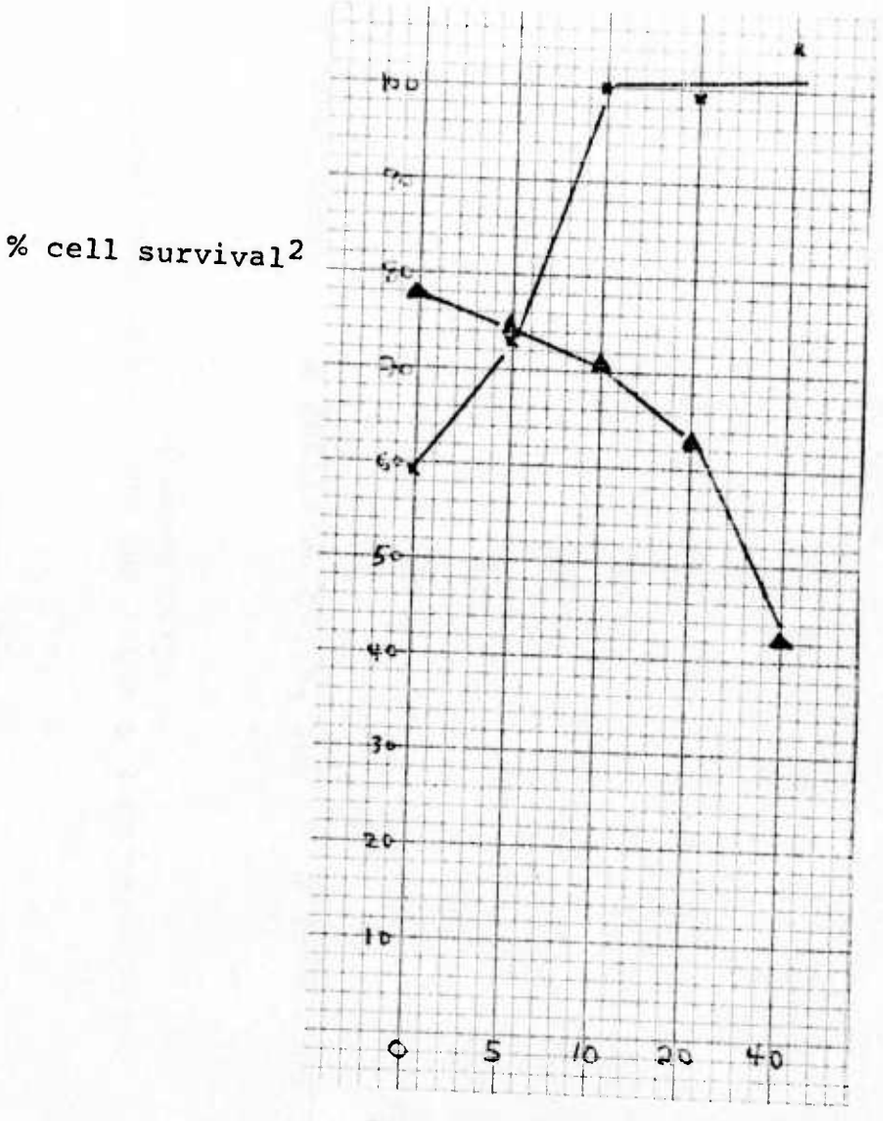
Dose of interferon[1]	% Surviving Cells[2] PR8	% Surviving Cells X-31
0	59	78
5	73	74
10	100	71
20	99	63
40	105	42

[1] PDD_{50} for VSV in human amnion cells

[2] Measured as neutral red uptake as described

Figure 6

Effect of Interferon Pre-Treatment
on the CPE of X-31 and PR8



[1]PDD_{50} for VSV in human amnion cells

[2]Measured as neutral red uptake as described

these cells over a wide range of concentrations. The rapid cell death will, of course, reduce the yield of virus from the infected cell and thus, may be viewed as a beneficial effect of interferon when considered from the point of view of a community of cells. Thus, under conditions of low multiplicity of infection (which corresponds to the physiological situation) the rapid death of those cells which become infected protects other cells from becoming infected subsequently by the yield from the former.

The effect appears to require fully infectious virus. Neither U.V.-inactivated virus, nor a PR8 strain of influenza which is unable to replicate in these cells manifests the toxic effect in interferon-treated cells. In addition, the effect is somewhat specific for influenza virus, as the toxic effects of neither NDV nor VSV are enhanced in interferon treated cells.

A similar effect has been seen with vaccinia, a DNA virus, but this is the first time such an effect has been observed for an RNA virus.

Virus cytotoxicity may be expressed in interferon-treated cells in one of two ways.

1. The toxic element may be a structural component of the virion itself, and need not be synthesized in the infected cell.

2. The toxic element is not a component of the virion, but its synthesis is not blocked in interferon-treated cells.

The results presented here tend to support the second alternative, since U.V.-irradiation eliminates the CPE. The synthetic double stranded RNA, Poly I:C is toxic for cells which have been exposed to interferon. It may be that a double-stranded RNA-intermediate is involved in the replication of influenza virus. Current work is directed toward dissecting the mechanism of the enhanced CPE of influenza in interferon-treated cells.

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